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G-quadruplex-induced instability during leading strand replication

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 25 July 2011

We have now received feedback from the reviewers of your manuscript, "G-quadruplex-induced instability during leading strand replication". As you will see from the comments copied below, all three referees consider your results an important advance and are in principle supportive of publication in The EMBO Journal. We should therefore be happy to consider a revised version further, pending satisfactory addressing of a number of specific issues detailed in the reports. These points mostly concern aspects of writing, discussion and interpretation, but referee 1 also raises some important points regarding statistical analysis of the data that would need to be satisfied. From an editorial point of view, I also need to ask you to amend the manuscript with an 'Author Contribution' statement, and to expand the currently minimal 'Material & Methods' section in the main manuscript text in accordance with our guide to authors that precludes moving of this section in (almost) its entirety into the supplement.

I am therefore returning the manuscript to you with the invitation to address these various points, hoping that you will be able to return a revised manuscript ideally within the next two weeks in order to facilitate timely publication. When preparing your letter of response, please be reminded that it will be necessary to diligently and comprehensively answer to all the points raised, and also bear in mind that this letter will form part of the Peer Review Process File available online to our readers in the case of publication (for more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html).

Should you have any further questions regarding your revision, please do not hesitate to get back to me directly. I am looking forward to receiving your revised manuscript.

Sincerely,

Editor
The EMBO Journal

_____

REFEREE REPORTS:

Referee #1 (Remarks to the Author):

Lopes et al. - EMBO J.

Nicolas and colleagues have previously shown that stability of the human CEB1 minisatellite in yeast cells depends upon the Pif1 helicase, which unwinds G4 DNA. This manuscript now provides a working model for how Pif1 performs this function in vivo. They use a simple, elegant approach, inserting the CEB1 repeat near an ARS element (ARS305), in both orientations, and then comparing instability in WT and pif1-delta cells. They demonstrate a clear orientation dependence for Pif1-dependent instability, which occurs when the G-rich sequence is the template for leading strand replication. This dependence is reversed when the proximal ARS is deleted, forcing most replication to initiate at a more distant site of opposite polarity. Instability is exacerbated by the G4-specific small molecule ligand, Phen-DC3. Analysis of replicating DNA by 2D gel electrophoresis identifies structures that form at CEB1 repeats in pif1-delta cells as similar to recombination intermediates, and shows that formation is dependent upon RAD51 and RAD52. These structures may be intermediates in repair, as while instability is diminished in rad51-delta or rad52-delta backgrounds, cell viability also drops. These results show that G4 DNA forms during transient denaturation of the duplex in the course of replication, that G4 DNA on the template for leading strand replication is normally unwind by PIF1, and that failed unwinding promotes recombination-dependent repair mediated by RAD51 and RAD52. Thus, this manuscript provides a novel and mechanistic view of replicative instability of G4 motifs, establishing when and where in the replication mechanism PIF1 functions to maintain their stability, and identifying functions for RAD51 and RAD52. The results presented here also provide strong support for the view that Phen-DC3 is stabilizing G4 structures in vivo, which will enable its use for future interesting analyses of sites, timing and conditions in which these structure form. The working model, based on the data, that provides a useful focus for future analysis of the mechanisms that stabilize and destabilize regions rich in G4 motifs.

The manuscript at hand goes significantly beyond a recent report (Paeschke et al. 2011) of genomewide analyses demonstrating enrichment of Pif1 at G4 motifs. The assays used by Paesche et al seem to have missed the striking orientation-dependence documented in the manuscript at hand and do not provide insights into how aberrant replication intermediates form.

The Nicolas manuscript provides a scholarly and balanced view of current understanding of genetics of stability of G4 motifs. However, there are some points at which the authors either overinterpret their own results or fail to draw distinctions that may prove to be important. These are noted below.

Throughout, the authors overstate the case that Pif1 alone promotes stability of G4 motifs and that Sgs1 or Rrm3 helicases do not contribute to G4 unwinding. It is true that mutations in the genes encoding Sgs1 and Rrm3 had no effect in these assays, but further experimentation (beyond the scope of this manuscript) will be necessary to show that this is generally true, especially taking into account the potential for in vivo redundancies and the fact that long G-rich sequences appear to be depleted from the yeast genome. In this vein, the Introduction makes an incorrect statement that Sgs1 unwinds G4 DNA in vitro "albeit at high concentration". In fact, Sgs1 would appear to be easily as robust as Pif1, as 1-2 nM Sgs1 unwound 50% of G4 DNA substrate (Sun et al. 1999), while approximately 10 nM Pif1 was required to reach the same endpoint (Ribeyre et al. 2009).

The use of G4 to describe G4 structures is jargony - this must be changed throughout.

p. 5: there appears to be a 10-fold difference in stability of CEB1-1.8 in wt and pif mutant cells, but the authors describe this as "remains stable". There is a much greater effect in the opposite orientation, but it is nonetheless important to know if this difference is statistically significant. Please include statistical analysis here and elsewhere wherever possible.

p. 5: I agree that the size variation is likely to evidence the independent origins of deletions, but it is possible that a single deletion renders the sequence susceptible to further events by different mechanisms and with different outcomes. (I do not favor this, but it is possible.) The authors might consider deferring this part of their conclusion to the Discussion.

Tables: could the tables be extended to include significance of other pairwise comparisons - e.g. p values for WT vs. pif1-delta, not just orientation I vs II?

The CEB1 repeat, because it is at a single site, offer an unusual potential to gather quantitative information on how G4 structures promote genomic instability. Would it be
possible to quantitate the 2D gel results to estimate what fraction of the cells form these unusual structures and relate these results to cell viability and to the fraction of cells exhibiting instability?

It was surprising that rad51-delta and rad52-delta strains showed so little instability in light of their several-fold reduced viability. These results raise the possibility that there may be factors that resolve other intermediates more efficiently than those generated by rad51 or 52. A comment would be useful.

Fig. 4: Why is the spot in left portion of diagonal bigger in orientation II than in orientation I?

The Introduction needs to state that Pfi1 and Sgs1 are both G4 helicases but of different families and of opposite directionality, and this should be stated as it is very likely to contribute to function in replication.

The Introduction needs to clarify whether the CEB1 construct is transcribed.

The use of asymmetrical to describe opposite replication orientation could be confusing. It might work well to use "orientation" uniformly throughout the ms, and to work the notion of orientation I and orientation II into a very early point of the manuscript, as that makes the experiments very clear.

The Discussion is long - it need not reiterate results so thoroughly, and it could discuss some issues better. For example, the authors should also comment on why the orientation-dependence was missed in the analysis by Paeschke et al. (2011); and on how their own results on orientation dependence may relate to the Kruisselbrink et al observations of directional instability at nematode FANCJ mutants.

Minor comments:

Abstract

Is cruciform formation or resolution dependent on Rad51/51 - please clarify

Rephrase: "instability relies on" as "instability occurs only if"

Introduction

Rephrasings recommended:
"scale" to "scale length"
"small change length" to "small length changes"
"code for polypeptides" to "encode polypeptides or portions of polypeptides"
"interstitial regions, and near or within coding regions"
"beginning uncover a direct role" to "uncovered a direct role of G4 in regulation of..."
"differentially upon" to "have different effects"
"led" to "enables"

The meaning of the clause beginning "somehow as a safeguard" is unclear, rewrite (possibly make the one long sentence into two).

State specifically that it is the nematode homolog of FANCJ that is critical for the instability at G4

motifs documented by Kruisselbrink et al.

Results

Explain CEB1-1.8 better in the text

p. 6 last paragraph: change "type" to "types"

p. 9: rephrase "also timely appear" as "appear with appropriate kinetics" or "in appropriate order for predicted order of replication"

p.9: change "independent on" to "independent of"

p.10: rephrase "the orientation I" change to "orientation I" here and elsewhere

Discussion
p. 11: change to "different types" (plural)

p. 13: change phrase to "but not Rrm3 or Sgs1" (inserting not, changing nor to or)

p. 13: it suggests.. that these last helicase MAY not....or act on the leading strand
- don't overdo it

p. 13: change "actors" to "factors"

p. 13: "improperly removed" - better "unresolved" or "persistent"

"initiating from such seed" - not common usage, clarify

Referee #2 (Remarks to the Author):

The Nicolas lab previously demonstrated a CEB1 mini-satellite array is mitotically unstable in yeast cells lacking the Rad27/FEN1 nuclease or Pif1 helicase, that mitotic instability is dependent on Rad51 and Rad52, and the ability of the CEB1 sequence to form G4 is required for instability. Here the authors show instability depends on the orientation of the array relative to the early-firing ARS305 origin in the pif1 mutant; instability increases by 28-fold when the G-rich sequence is the leading strand template, compared with when it is the lagging strand template. This bias can be reversed by mutating ARS305 so the array is replicated by ARS306 placing the G-rich sequence on the lagging strand template. Although the array is stable in wild type, the Phen-DC3 G4 stabilizing ligand induces greater instability when the G-rich sequence of the array is the leading strand template, consistent with the pif1 data. Furthermore, by 2D gel analysis, Rad51/Rad52-dependent X-forms are observed in the pif1 mutant, or in wild type treated with Phen-DC3, only when the G-rich sequence is on the leading strand template. Together, these data provide novel insight into the mechanisms of instability induced by G4 secondary structures and the role of Pif1 in resolving them.

This study offers additional insights into Pif1 function independent of the study recently published by the Zakian lab. The CEB1 array has been extensively characterized by the Nicolas lab and results in extremely high levels of rearrangements dependent on the number of repeats. In addition to analyzing pif1 mutants, all of the results are verified in wild type in the presence of the G4 stabilizing ligand. The suppression of these events by rad51 and rad52 mutations is also a unique aspect of the Nicolas study. The most interesting and surprising finding is the orientation dependence for instability and aberrant structures detected by 2D gels in the pif1 mutant. It is unclear why no orientation effect was observed for recombination induced by G4 sequences in pif1 mutants by Paeschke et al (2011), but this could be due to the location and less well characterized flanking ARS elements in that study.

Minor comments
p. 5, line 5 from bottom: 100-fold is missing.
p. 10. Are the rearrangements found in rad51 and rad54 mutants deletions? If so, it would suggest

they occur by SSA.
The Bzymek ref is incorrect, it should be 2010.

Referee #3 (Remarks to the Author):

In this work, Lopes et al demonstrate orientation-dependent instability resulting from the presence of the G-rich CEB1 DNA sequence in yeast. Since the work is an extension of previous studies (Piazza et al 2010: Ribeyre et al., 2009), showing significant genome instability associated with CEB1, the novelty of the present study rests upon the location of CEB1 in the leading strand and upon the links between the instability of CEB1 and the absence of Pif1 helicase. I think both of these points are well demonstrated in the manuscript. Since Pif1 is thought to play a role in the removal of G4 DNA, and CEB1 is presumed to be forming such tetraplex structures, the proposal is that G4 DNA is problematic upon encounter by a progressing replication fork. As such, the resulting genome rearrangements could have evolutionary implications. The work presented in the manuscript is interesting, and I would recommend it for publication in EMBO Journal.

Whether or not G4 DNA forms in cells is controversial, and while some workers in this field are 'believers' many others are not. Therefore any concrete evidence for the formation of G4 DNA by CEB1 (rather than it simply forming secondary structures that might induce slippage) needs to be detailed in a revised manuscript. In my opinion neither the use of Phen-DC3 nor mutated CEB1 sequences eliminate the possibility that the instability reported with CEB1 is not due to undefined DNA configurations rather than G4 DNA. Blocks to replication fork progression by mechanisms other than G4, followed by fork processing, would be expected to lead to the observed Rad51-Rad52 dependent X-molecules, so this aspect of the work is less novel than suggested.

The model presented in Figure 5 is similar to models previously proposed by workers in the telomerase field.

Finally, the work is not helped by the fact that the manuscript is poorly written and difficult to read (particularly the introduction). In addition, the manuscript as a whole is overly long given that there is not so much data. Some aspects are carelessly presented (grammatical errors, spelling errors, reference citations without the year etc).

1st Revision - authors' response 03 August 2011

Please find below our detailed answer to your and the reviewers questions and suggestions.
Main changes are the following. Material and Methods as been moved to the main text, except Method of strain construction which is rather long and not immediately necessary to follow the main text. In the Introduction (several instances p.4) and Discussion (p.13), we added precise statements concerning the relevant Paeschkeís 2011 data, all being done in a constructive way to show complementarities and differences. All statistical analyses were provided in Table 1 and 2 legends. We added the key ones in the Results section (p.6). We added one interesting reference ´ De S. and Michor, F. (2011) DNA secondary structures and epigenetic determinants of cancer genome evolution. Nat. Struc. & Mol. Biol. Advanced on line 3 July 2011; doi:10.1038/nsmb.2089". Author contribution is provided. Figures were not changed.

Referee #1 (Remarks to the Author):
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small molecule ligand, Phen-DC3. Analysis of replicating DNA by 2D gel electrophoresis identifies structures that form at CEB1 repeats in pif1-delta cells as similar to recombination intermediates, and somehow that formation is dependent upon RAD51 and RAD52. These structures may be intermediates in repair, as while instability is diminished in rad51-delta or rad52-delta backgrounds, cell viability also drops. These results show that G4 DNA forms during transient denaturation of the duplex in the course of replication, that G4 DNA on the template for leading strand replication is normally unwind by PIF1, and that failed unwinding promotes recombination-dependent repair mediated by RAD51 and RAD52. Thus, this manuscript provides a novel and mechanistic view of replicative instability of G4 motifs, establishing when and where in the replication mechanism PIF1 functions to maintain their stability, and identifying functions for RAD51 and RAD52. The results presented here also provide strong support for the view that Phen-DC3 is stabilizing G4 structures in vivo, which will enable its use for future interesting analyses of sites, timing and conditions in which these structure form.
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Throughout, the authors overstate the case that Pif1 alone promotes stability of G4 motifs and that Sgs1 or Rrm3 helicases do not contribute to G4 unwinding. It is true that mutations in the genes encoding Sgs1 and Rrm3 had no effect in these assays, but further experimentation (beyond the scope of this manuscript) will be necessary to show that this is generally true, especially taking into account the potential for in vivo redundancies and the fact that long G-rich sequences appear to be depleted from the yeast genome.

-> Several hypotheses to explain the absence of effect of Rrm3 and Sgs1 are provided in the Discussion p.13 and p.14

In this vein, the Introduction makes an incorrect statement that Sgs1 unwinds G4 DNA in vitro "albeit at high concentration". In fact, Sgs1 would appear to be easily as robust as Pif1, as 1-2 nM Sgs1 unwound 50% of G4 DNA substrate (Sun et al. 1999), while approximately 10 nM Pif1 was required to reach the same endpoint (Ribeyre et al. 2009).
-> p4, we now say: In S. cerevisiae, the Sgs1 (RecQ ortholog) and Pif1 helicases which belong to different helicase sub-families and work in opposite directionality (3í-5í and 5-3í, respectively) unwind G-quadruplexes in vitro (Ribeyre et al., 2009; Sun et al., 1999).

The use of G4 to describe G4 structures is jargony - this must be changed throughout.

-> Done

p. 5: there appears to be a 10-fold difference in stability of CEB1-1.8 in wt and pif mutant cells, but the authors describe this as "remains stable". There is a much greater effect in the opposite orientation, but it is nonetheless important to know if this difference is statistically significant. Please include statistical analysis here and elsewhere wherever possible.

-> All pairwise combinations of statistical analyses (p-values) were indicated in the text and/or in Table 1 and 2 Legends. The requested p-values are now stated in the text (p.6)

p. 5: I agree that the size variation is likely to evidence the independent origins of deletions, but it is possible that a single deletion renders the sequence susceptible to further events by different mechanisms and with different outcomes. (I do not favor this, but it is possible.) The authors might consider deferring this part of their conclusion to the Discussion.

-> We agree with the reviewer that the step-wise molecular origin of these complex rearrangements remains to be established. Here and in previous studies, Independently of the frequency of CEB1 rearrangements, we always observed such size and sequence diversity. Indeed, it might occur in a single step or possibly, as stated, in two steps i.e; one master deletion step and then diversification by another mechanism as envisaged by the reviewer. The fact that all events are Rad51 and Rad52-dependent and the lack of de novo mutations favor a single step mechanism. Thus, our current parsimonious model is the occurrence of a single but complex SDSA event (Lopes et al., 2006). Perhaps, in the future, the high frequency of events now reached will allow us to decipher the molecular steps and intermediates leading to the rearrangements.

Would it be possible to quantitate the 2D gel results to estimate what fraction of the cells form these unusual structures and relate these results to cell viability and to the fraction of cells exhibiting instability?

-> The measurement of the X-spike is normalized to the Y arc signal. This internal normalization provides an estimate of the relative accumulation of the molecules in the X-spike in one condition versus another (WT vs. pif1delta, orientation I vs. II). However, it does not give a quantitative estimation in term of cells forming this intermediate simply because the half-life of the intermediates in the X-spike is likely not the same as the one (the replication fork) in the Y-arc. Thus it cannot be safely compared to the cell viability and CEB1 instability measurements.

It was surprising that rad51-delta and rad52-delta strains showed so little instability in light of their several-fold reduced viability. These results raise the possibility that there may be factors that resolve other intermediates more efficiently than those generated by rad51 or 52. A comment would be useful.

-> The viability is reduced about 25% in the orientation I in the pif1rad51delta double ñmutant, compared to orientation II or single mutants (or WT). We proposed that Rad51 and Rad52 acts downstream of the replication block by the G4 to bypass it. The homologous recombination (the process that generates the spike by 2D-gel and the CEB1 rearrangements) allows the G4 bypass and fork restart. In its absence, nor the rearrangements nor the spike are seen, and because of this viability drop, we propose that cells cannot restart the fork, and stop to divide.
p.13, we clarify this point by adding "otherwise leading to cell death" after "G-quadruplexes formed by CEB1 will remain unprocessed and channeled into the recombinational-repair pathway"

Fig. 4: Why is the spot in left portion of diagonal bigger in orientation II than in orientation I?
->The stronger signal observed in the diagonal of the gels WT-CEB1 orientation II in the Figure 4A (but not in Figure 4D, same strain and restriction enzymes used) is likely to be a small fraction of partially digested DNA, that should normally migrate in the 1N spot.

The Introduction needs to state that Pfi1 and Sgs1 are both G4 helicases but of different families and of opposite directionality, and this should be stated as it is very likely to contribute to function in replication.

->Added in p.4.

The Introduction needs to clarify whether the CEB1 construct is transcribed.

->Informations about the structure of CEB1-1.8 allele have been added in the results section p. 5. The last ß of the Introduction states that the site of insertion was chosen to avoid the CEB1 construct to be transcribed. The orientation-dependent instability inversion observed in the ars305-deleted strains argues that the main process responsible for the array instability is replication, not transcription.

The use of asymmetrical to describe opposite replication orientation could be confusing. It might work well to use "orientation" uniformly throughout the ms, and to work the notion of orientation I and orientation II into a very early point of the manuscript, as that makes the experiments very

clear.

-> Done

The Discussion is long - it need not reiterate results so thoroughly, and it could discuss some issues better. For example, the authors should also comment on why the orientation-dependence was missed in the analysis by Paeschke et al. (2011); and on how their own results on orientation dependence may relate to the Kruisselbrink et al observations of directional instability at nematode FANCJ mutants.

-> Done in several instance in the Introduction (p.4) and in the Discussion (p.13)

Modifications corresponding to "minor comments" have been introduced.

Referee #2 (Remarks to the Author):

The Nicolas lab previously demonstrated a CEB1 mini-satellite array is mitotically unstable in yeast cells lacking the Rad27/FEN1 nuclease or Pif1 helicase, that mitotic instability is dependent on Rad51 and Rad52, and the ability of the CEB1 sequence to form G4 is required for instability. Here the authors show instability depends on the orientation of the array relative to the early-firing ARS305 origin in the pif1 mutant; instability increases by 28-fold when the G-rich sequence is the leading strand template, compared with when it is the lagging strand template. This bias can be reversed by mutating ARS305 so the array is replicated by ARS306 placing the G-rich sequence on the lagging strand template. Although the array is stable in wild type, the Phen-DC3 G4 stabilizing ligand induces greater instability when the G-rich sequence of the array is the leading strand template, consistent with the pif1 data. Furthermore, by 2D gel analysis, Rad51/Rad52-dependent X-forms are
observed in the pif1 mutant, or in wild type treated with Phen-DC3, only when the G-rich sequence is on the leading strand template. Together, these data provide novel insight into the mechanisms of instability induced by G4 secondary structures and the role of Pif1 in resolving them.

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-> This point is discussed (see Response to referee#1).

-> Modifications corresponding to "minor comments" have been introduced.

Referee #3 (Remarks to the Author):

In this work, Lopes et al demonstrate orientation-dependent instability resulting from the presence of the G-rich CEB1 DNA sequence in yeast. Since the work is an extension of previous studies (Piazza et al 2010: Ribeyre et al., 2009), showing significant genome instability associated with CEB1, the novelty of the present study rests upon the location of CEB1 in the leading strand and upon the links between the instability of CEB1 and the absence of Pif1 helicase. I think both of these points are well demonstrated in the manuscript. Since Pif1 is thought to play a role in the removal of G4 DNA, and CEB1 is presumed to be forming such tetraplex structures, the proposal is that G4 DNA is problematic upon encounter by a progressing replication fork. As such, the resulting genome rearrangements could have evolutionary implications. The work presented in the

manuscript is interesting, and I would recommend it for publication in EMBO Journal.

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The model presented in Figure 5 is similar to models previously proposed by workers in the telomerase field.

Finally, the work is not helped by the fact that the manuscript is poorly written and difficult to read (particularly the introduction). In addition, the manuscript as a whole is overly long given that there is not so much data. Some aspects are carelessly presented (grammatical errors, spelling errors, reference citations without the year etc).

-> Evidence that CEB1 forms G-quadruplex in vivo are perhaps best of any G-quadruplex studied so far as it relies on mutational analyses and chemical interference with the highly specific G-quadruplex ligand of the Phen-DC bisquinolinium series. In vitro data on CEB1 G-quadruplex formation, interaction with Phen-DC and unwinding by the purified Pif1 protein have been reported (Ribeyre et al., 2009; Piazza et al., 2010). The striking parallel behavior of CEB1 in pif1 and WT + Phen-DC is strong to start "to believe" in the in vivo formation of G-quadruplex in the present case.

Acceptance letter 09 August 2011

Thank you for submitting your revised manuscript for our consideration. I have now had a chance to look through it and to assess your responses to the comments raised by the original reviewers, and I am happy to inform you that there are no further objections towards publication in The EMBO Journal.

We should now have everything we need for production, and you shall receive a formal letter of acceptance shortly.

Sincerely,

Editor
The EMBO Journal

